# On-line Capillary Separations/Tandem Mass Spectrometry for Protein Digest Analysis by Using an Ion Trap Storage/Reflectron Time-of-Flight Mass Detector

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Capillary separations interfaced to tandem mass spectrometry provide a very powerful tool for the characterization of biological macromolecules such as proteins and peptides. The development of real time data-dependent data acquisition has further enhanced the capability of this method. However, the application of this technique to fast capillary separations has been limited by the relatively slow spectral acquisition speed available on scanning mass spectrometers. In this work, an ion trap storage/reflectron time-of-flight mass spectrometer (IT/reTOF-MS) has been used as an on-line tandem mass detector for capillary high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) separations of peptide mixtures including a protein digest. By taking advantage of the nonscanning property of the time-of-flight mass spectrometer, a fast spectral acquisition rate has been achieved. This fast spectral acquisition rate, combined with a new protocol that speeds up tickle voltage optimization, has provided MS/MS spectra for multiple components in a hemoglobin digest during one liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) run. Further, the IT/reTOF-MS has the speed to provide MS/MS spectra for multiple components in a CE separation of a synthetic peptide mixture within one CE/MS/MS run. (J Am Soc Mass Spectrom 1997, 8, 1237–1246) © 1997 American Society for Mass Spectrometry

The combination of capillary separation methods and mass spectrometry provides enormous capabilities for the analysis of minute quantities of analytes in very complex mixtures [1–3]. It is an extremely useful technique for the characterization of biological macromolecules in very complex biological matrices, where high separation efficiency and precise identification and structural elucidation of the analytes are required with limited sample. As a result, on-line capillary liquid chromatography/mass spectrometry (LC/MS) and capillary electrophoresis (CE)/MS have been widely used to solve a variety of difficult biomedical problems [4].

Whereas these on-line LC/MS and CE/MS methods have been successful in certain applications based on the analyte retention times and molecular weight obtained, this information is often insufficient, especially when compared to the enormous amount of information that can be obtained by tandem mass spectrometry (MS/MS) [5, 6]. Tandem mass spectrometry allows for the structural elucidation and the identification of the analytes based upon molecular weight and also on the resulting fragmentation pattern. This method is especially useful in protein structure analysis, where the structural information can be used to pinpoint the locations of posttranslational modifications or mutation sites of low-level cellular proteins [7, 8].

The interface of tandem mass spectrometry to LC and CE separations has recently been reported for the structural analysis of proteins and peptides [9, 10]. The direct coupling of separation methods to tandem mass spectrometry has considerably reduced sample consumption by eliminating losses occurring in the sample collection and transfer steps. However, in most of these experiments, only the MS/MS spectrum of one component was obtained for each separation run. Multiple separation runs were required in order to obtain the MS/MS spectra for multiple components, resulting in a dramatic increase in sample consumption and analysis time. Recently, Yates et al. [11] and Lee et al. [12] reported data-dependent data acquisition in real time for collecting MS/MS spectra from LC separations by using triple quadrupole instruments that allowed for

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acquiring MS/MS spectra of multiple components during one separation run. As an effort to develop their commercial product, Finnigan MAT has combined real time data analysis and data acquisition in their ion trap mass spectrometers for on-line LC/MS/MS experiments [13].

In addition to the difficulties associated with the development of software and hardware that are used to perform the real time data analysis and data acquisition for on-line MS/MS experiments, the greatest challenge, however, lies in the spectral acquisition speed required for high-resolution separations. As a result of the high efficiency and speed of capillary separation methods, the peak widths are extremely narrow. In CE separations, for example, the peak width is usually on the order of several seconds. It is thus difficult to achieve sufficiently rapid scans for each peak by using conventional scanning mass spectrometers such as quadrupole mass spectrometers. A substantial fraction of an analyte peak could have already eluted before the data is analyzed and the system is optimized. For ion trap mass spectrometers, although they can scan at a fast rate, only limited multiple scans over a chromatographic peak can be achieved, and mass resolution and accuracy are also sacrificed. In addition, multiple data-acquisition and data analysis steps are required to optimize the fragmentation efficiency in the ion trap, because the optimal tickle voltage range is much narrower than that in the triple quadrupole instruments, where an acceleration potential is used to enhance collision induced dissociation (CID) [14].

In previous work, we have demonstrated the procedures for performing MS/MS experiments of synthetic peptides on an ion trap storage/reflectron time-of-flight mass spectrometer (IT/reTOF-MS) by using a direct loop injection method [15]. The IT/reTOF-MS uses a quadrupole ion trap as a front end storage device that converts a continuous electrospray beam into pulsed ion packets for mass analysis by a reflectron time-of-flight mass spectrometer (TOF-MS). In the MS/MS mode, the precursor ion is isolated and activated in the ion trap and its product ions are extracted by a dc pulse into the TOF-MS for mass analysis. The use of TOF-MS for mass detection eliminates the scanning step used in the ion trap spectrometer, and thus significantly increases the sampling speed and duty cycle for on-line separations. This device also has some additional advantages including the fact that the dc extraction is monodirectional versus the bidirectional ejection in conventional ion trap mass spectrometers that results in improved sensitivity. Also, the effect of space charge on mass accuracy and resolution in the IT/reTOF-MS is not as severe as that in the ion trap mass spectrometer, because the ion ejection is no longer based on the resonant frequency.

In this work, we report the development and performance of on-line MS/MS experiments for capillary separations by using the IT/reTOF-MS detector. Ion isolation was performed by a broadband excitation method using the stored waveform inverse Fourier transform (SWIFT) technique [16]. In order to optimize on-line the tickle voltage, a data-dependent data acquisition protocol was developed that employed two different tickle voltage optimization schemes. In the first scheme, the intensity ratio of the molecular ion peaks in two contiguous mass spectra was used to determine if the optimal tickle voltage was achieved. These two contiguous mass spectra were obtained with tickle voltage on and off, respectively. In the second scheme, the intensity ratio of the product ions over the molecular ion within one spectrum was used to determine the optimal tickle voltage. The second scheme reduced data analysis time by 50% compared to the first scheme. Therefore, it is especially useful for on-line optimization of MS/MS experiments for fast separations or separations of complex mixtures that contain co-eluting components. In this article, we report the development and the use of IT/reTOF-MS as a tandem mass detector to obtain MS/MS spectra for multiple components from an LC separation of a protein digest and CE separation of a synthetic peptide mixture. The design and performance of the SWIFT isolation waveform, and the procedures for the use of the first scheme to obtain MS/MS spectra from on-line capillary high-performance liquid chromatography (HPLC) separation of synthetic peptide mixtures will be provided in a separate report [17].

## Experimental

### *Liquid Chromatography*

Capillary HPLC columns (0.25 i.d. × 0.36 o.d. × 200 mm) were packed in-house with 5 μm C-18 silica gel by using a slurry packing method. Separations were performed using a Star 9012 solvent delivery system (Varian Associates, Inc., Walnut Creek, CA) which was operated at a constant flow rate of 200 μL/min. The solvent split was performed by a prime/purge valve located immediately before the injection valve with a split ratio of 39:1, resulting in a flow rate of 5 μL/min. UV detection was performed by using a Star 9050 (Varian) variable wavelength UV detector with a micro flow cell (Varian) at 214 nm. Gradient elution was performed by using 0.1% trifluoroacetic acid (TFA) in water as solvent A and 0.1% TFA in acetonitrile as solvent B. Sample stacking was performed by running solvent A for 2 min before the start of the gradient.

### *Capillary Electrophoresis*

The capillary electrophoresis apparatus was built in-house. Fused silica capillaries from Polymicro Technologies, Inc. (Phoenix, AZ) were used as separation capillaries, whose inner surface was coated with polybrene, which reversed the charge on the inner capillary wall and reduced peptide adsorption. A high voltage power supply (Model CZE 1000R, Spellman, Plainview, NY) operated in the negative mode was used to provide the

separation voltage. A 50 mM formic acid solution supplemented with 5 mM ammonium acetate was used as the running buffer. On-column detection was performed by using a variable wavelength UV detector (Model SC 100, ThermoSeparation Products, Fremont, CA) at 198 nm. A nanospray source was constructed in house in which the outlet end of the CE capillary was directly used as the electrospray needle. The polymer coating of the capillary tip was first burnt off, then the tip was etched by concentrated hydrofluoric acid, and was finally electroless plated with silver [18].

### *Mass Spectrometer*

The IT/reTOF-MS has been described in detail in previous work [18, 19]. It consists of a differentially pumped reflectron time-of-flight mass spectrometer (Model D-850) interfaced to a quadrupole ion trap storage device (Model C-1251, R. M. Jordan Co., Grass Valley, CA). Ion beams generated from the electrospray source were introduced into an atmospheric pressure interface by a 0.5-mm-i.d. stainless steel capillary heated to 120°C. The interface region was evacuated to a pressure of less than 1 torr, where a cylindrical lens and a 325 $\mu$m orifice skimmer were aligned co-axially. The cylindrical lens serves to focus the ion beam into the skimmer orifice. The ions traversing through the skimmer were focused into the ion trap by an Einzel lens. The ions were stored in the ion trap for a preset period of time (typically from 0.25 to 0.5 s, corresponding to a sampling rate of 2 to 4 Hz) and with a preset radiofrequency (rf) voltage (typically from 1000 to 1500 $V_{pp}$). After the ions were stored for a preset time, a dc pulse (−400 V, 2 $\mu$s pulse width) was applied to the exit endcap of the ion trap and ions were extracted into the time-of-flight device for mass analysis.

### *Selection of the Precursor Ion*

All samples were first subjected to an LC/MS or CE/MS analysis, from which the elution order of the peaks were determined. For co-luting components and components that exhibited more than one charge state, this initial separation allowed for selecting the precursor ion. Multiply charged ions were favored over their singly charged counterparts wherever possible, because they undergo fragmentation more readily. The $m/z$ values of the ions were entered into a user-written data analysis program according to their elution order. In the LC/MS/MS run, the integrated peak intensities of these $m/z$ values were monitored in the collected mass spectra. If a preset threshold was reached, which indicated the elution of a peak, the data analysis program immediately delivered a command through a digital input/output board (CIO-DIO 24, Computer Boards, Inc.) to a user-written waveform generation program that was operating on a second computer. Upon receiving the command, the waveform generation program generated isolation and activation waveforms.

### *Isolation of the Precursor Ion*

The ion isolation was performed by using the SWIFT technique [16]. Because the on-line MS/MS analyses in these experiments were performed under the condition of $q_z < 0.4$, the $\beta_z$ values of the precursor ions were calculated by using the approximation equation $\beta_z = [a_z + (q_z^2/2)]^{1/2}$ [20], and the secular frequency was calculated as $f_z = \beta_z\Omega/(2\pi)$. A frequency spectrum covering a broad frequency range from 10–200 kHz was first generated with a notch window centered at the secular frequency. This frequency spectrum was then converted into a time-domain waveform by using the inverse Fourier transform. A quadratic phase modulation method similar to that reported by Chen et al. [21] was used in these experiments to scramble the initial phases in the frequency spectrum. All the above operations were performed on a Pentium 200 computer (Gateway 2000, North Sioux City, SD). After the appropriate SWIFT waveform was constructed on the computer, it was loaded onto an arbitrary waveform generator (AWFG) board (Model PCIP-AWFG, Keithley Metrabyte, Taunton, MA) embedded in this computer. The AWFG board then generated the analog signal of this waveform which was applied to one of the endcaps of the ion trap.

### *Activation of the Precursor Ion*

Both helium and argon have been tested as the buffer gas in our experiments, and their effect on the fragmentation patterns and efficiency will be reported in detail elsewhere [18]. For the experiments reported herein, argon was used as the buffer gas. The tickle voltage for each precursor ion was optimized according to the following scheme: a minimum tickle voltage ($V_{min}$), a maximum tickle voltage ($V_{max}$), and an initial tickle voltage ($V_{ini}$) were set for each of the peptides (usually at 0.5, 3.5, and 2.0 $V_{p\text{-}p}$, respectively). After the ion isolation step and a short cooling period (5 ms), $V_{ini}$ was applied on the endcap, and the ratio ($R$) of the product ions intensity ($I_{pt}$) over the precursor ion intensity ($I_{pr}$) was calculated from the resulting mass spectrum:

$$R = \frac{I_{pt}}{I_{pr}}$$

If $R$ was found to be above a preset range, a new tickle voltage ($V_2$) was generated to acquire the next MS/MS spectrum:

$$V_2 = \frac{V_{ini} + V_{min}}{2}$$

A new ratio ($R'$) was calculated from this spectrum, and its value was compared with the preset range and the next tickle voltage ($V_3$) was generated based on the result of the comparison.

$$V_3 = \frac{V_2 + V_{min}}{2}$$

if $R'$ was above the preset range, or

$$V_3 = \frac{V_{ini} + V_2}{2}$$

if $R'$ was below the preset range.

This process was repeated until $R$ fell into the preset range. If $R$ was found to be below a preset range, after trying with the initial tickle voltage, $V_2$ was generated according to

$$V_2 = \frac{V_{ini} + V_{max}}{2}$$

A similar process was repeated until $R$ fell into the preset range.

### *Detection of the Product Ions*

After the ion activation period, the ions were cooled down in the trap by the buffer gas for about 20 ms before a dc pulse was applied to one of the endcaps of the ion trap to extract the product ions into the TOF-MS for mass analysis.

### *Materials*

All peptide and protein samples, ammonium acetate, acetonitrile, and formic acid were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Trypsin was purchased from Promega (Madison, WI). Water used to prepare CE separation buffers and HPLC solvent was generated with a Milli-Q water purification system (Millipore Corp., Bedford, MA). For tryptic cleavages, a 100 $\mu$g aliquot of pig hemoglobin was incubated for 21 h at 37°C with a protein to enzyme ratio of 30:1 (w/w) in 50 mM $NH_4HCO_3$ solution at pH 8.2. The digested materials were then spin-dried under vacuum to remove the salt and reconstituted in water to a concentration of $2 \times 10^{-5}$ M of original protein.

## Results and Discussion

The high separation efficiency and speed of capillary separation methods, combined with the structural elucidation capability of tandem mass spectrometry, are methods of choice for the structural analysis of minute amounts of protein. In these experiments, a small quantity of isolated protein is first subjected to chemical or enzymatic digestion. The digest products are then loaded onto a capillary separation column and are detected by a tandem mass spectrometer, which provides the precise information in terms of peptide sequences and modification sites. A high column separation efficiency is essential in these experiments, because these digests are usually very complex, containing tens of fragments. The narrow peak widths resulting from the high separation efficiency, however, also provide a challenge for an on-line tandem mass spectrometer. A cycle consisting of spectral acquisition, data analysis, and system optimization must be repeated several times before the mass detector can collect optimal MS/MS spectra. These events must occur within the short time duration of peaks eluting from the separation. The purpose of this work is to demonstrate that the required rapid spectral acquisition speed can be achieved in the IT/reTOF tandem mass spectrometer configuration. This high speed, combined with a fast data-dependent data acquisition scheme, allows for obtaining MS/MS spectra for multiple components from on-line capillary LC or CE separations.

**Table 1.** Measured intensity ratio of product ions over precursor ion for several peptides

| Peptides | MW | Charge state of the precursor ion | Intensity ratio |
|---|---|---|---|
| Leucine enkephalin | 555.6 | I | 23.5 |
| Methionine enkephalin-Arg-Phe | 877.0 | I | 19.6 |
| Angiotensin III | 931.1 | II | 17.2 |
| Bradykinin | 1060.2 | II | 19.8 |
| Angiotensin I | 1296.5 | II | 21.3 |
| Neurotensin | 1672.9 | III | 18.5 |

### *Determination of the Optimal Intensity Ratio*

The intensity ratio of product ions over the precursor ion plays a critical role in obtaining optimal MS/MS spectra. This ratio was determined by calculating the intensity ratio in the MS/MS spectra of a variety of synthetic peptides. These MS/MS spectra were obtained by manually optimizing the tickle voltage with direct infusion of the peptide samples. The intensity ratios of these peptides are listed in Table 1.

Most of the peptides tested have an intensity ratio around 20. In order to achieve the MS/MS spectra from an on-line separation of real samples, such as a protein digest, where the determination of the intensity ratio of an individual component is not possible, a universal intensity ratio is required. In these experiments, a ratio of 20 was used as the optimal value. Practically, it is almost impossible to optimize the intensity ratio to a specific value. Therefore, a certain range around the optimal value is necessary. A narrow range allows the system to reach a more accurate optimal tickle voltage, but this requires a longer time. A wide range allows the system to reach the final tickle voltage more quickly, but often at the expense of tickle voltage accuracy. In these experiments, a range from 19 to 21 was used that required a change in the tickle voltage of 7 to 8 times before reaching the optimal value.

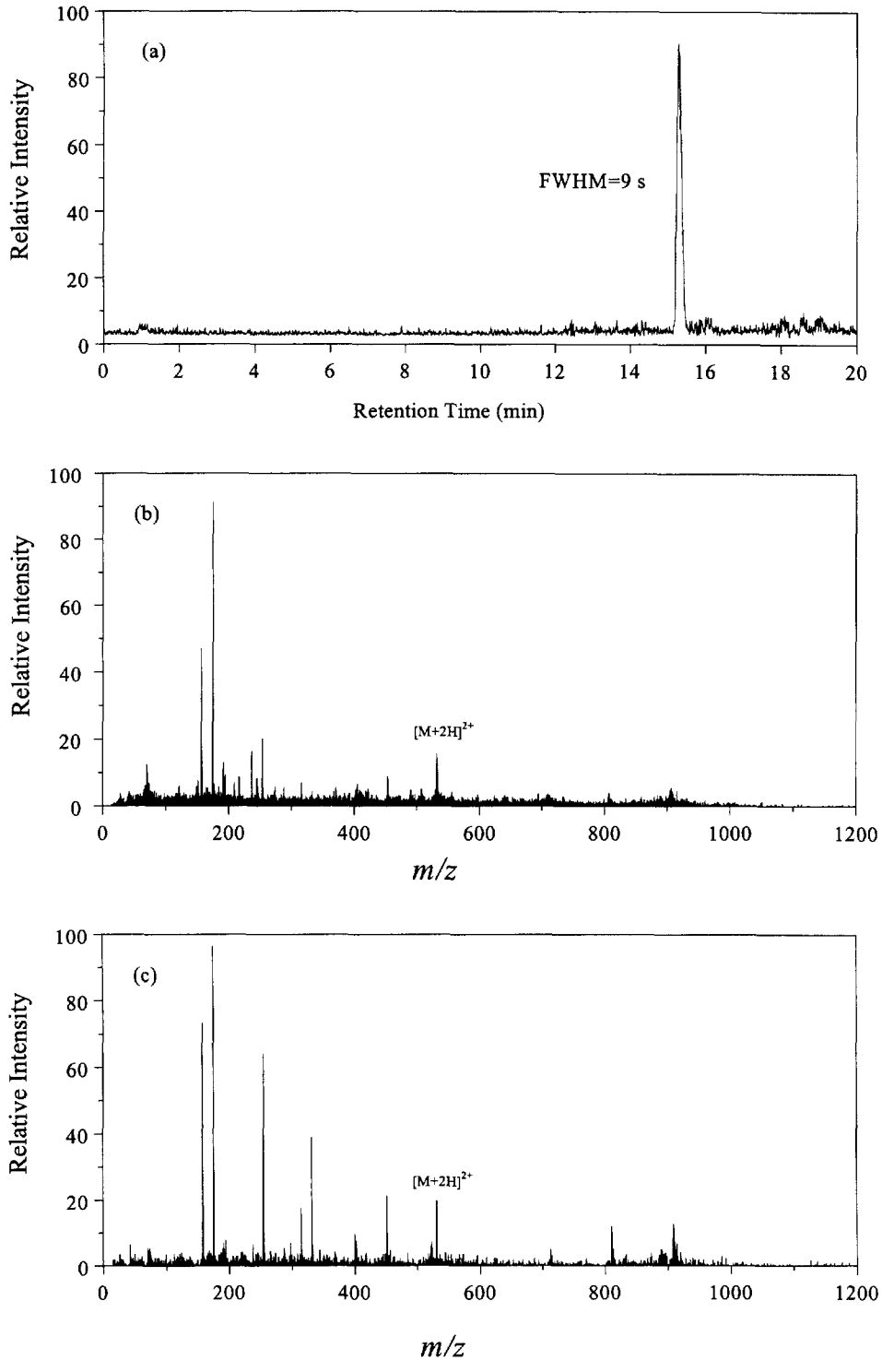


**Figure 1.** MS/MS spectra of bradykinin obtained from capillary HPLC separation. (**a**) TIC of the elution profile of bradykinin, with a FWHM of 10 s; (**b**) MS/MS spectrum obtained by using intensity ratio from two contiguous spectra; (**c**) MS/MS spectrum obtained by using intensity ratio within one spectrum. Doubly charged ion of bradykinin was selected as the precursor ion.

## *Performance of Data-Dependent Data Acquisition*

In order to collect MS/MS spectra for multiple components from an on-line separation, a tandem mass detector must be capable of adjusting its operating parameters as different components elute from a column. This adjustment can only be made after analyzing the collected spectra. Therefore, real-time data-dependent data acquisition is required for a tandem mass detector for on-line separations. Because of the narrow peak widths

**Table 2.** Measured optimized tickle voltages for bradykinin with different sample injection amounts

| Sample injection amount (pmol) | FWHM (s) | Tickle voltage ($V_{p\text{-}p}$) |
|---|---|---|
| 25 | 11 | 1.615 |
| 10 | 9 | 1.610 |
| 5 | 7 | 1.610 |

achieved in fast capillary separations, the data analysis and operating parameter adjustment should be accomplished in a very short time. For CID in the ion trap, the fine adjustment of the tickle voltage is critical, because only a very narrow range (usually ±0.01 V) of the voltage can result in efficient fragmentation of the precursor ion. The result is that a cycle containing data analysis and tickle voltage optimization must be repeated several times before the tickle voltage can be optimized.

In order to reduce the time required for data analysis and tickle voltage optimization, efforts have been made in our experiments to develop a more efficient data analysis scheme. In a previous version of our data-dependent data acquisition scheme, the intensity ratio of the molecular ion peaks in two contiguous mass spectra was used as a criterion for tickle voltage optimization. These two contiguous mass spectra were obtained with tickle voltage on and off, respectively. Generally, the cycle consisting of data analysis and tickle voltage adjustment is repeated 7 to 8 times before the tickle voltage is optimized, so that 7 to 8 s are required for this period with a mass sampling rate of 2 Hz. Therefore, for a peak which has a peak width full width at half maximum (FWHM) of less than 10 s, a relatively large portion of the peak has been eluted before the mass detector starts to collect optimal MS/MS spectra. In this work, a data-dependent data analysis scheme that employs the use of the intensity ratio of the product ions over the molecular ion within one spectrum has been used to optimize the tickle voltage. Although the number of optimization cycles repeated remains the same, the optimization time can

**Table 3.** Comparison of calculated and measured tryptic fragments of pig hemoglobin from capillary HPLC/MS analysis

| No. | Fragment | Retention time (min) | Calcd. mass | Measd. mass[a] | Tickle voltage (V) | Sequence |
|---|---|---|---|---|---|---|
| αT1 | 1–7 | 18.7 | 702.8 | 702.7 | 1.942 | VLSAADK |
| αT2 | 8–11 | 17.6 | 430.5 | 430.6 | — | ANVK |
| αT3 | 12–16 | 17.8 | 531.6 | 531.5 | 1.396 | AAWGK |
| αT4 | 17–31 | 27.4 | 1422.5 | 1422.8 | 1.658 | VGGQAGAHGAE ALER |
| αT5 | 32–40 | 23.1 | 1041.3 | 1041.2 | — | MFLGFPTTK |
| αT6 | 41–56 | 26.3 | 1877.0 | 1877.3 | — | TYFPHFNLSHGS DQVK |
| αT7 | 57–61 | 18.1 | 539.6 | 539.5 | 1.514 | AHGQK |
| αT8 | 62–68 | 19.2 | 716.8 | 716.9 | 1.476 | VADALTK |
| αT9 | 69–90 | 34.5 | 2237.5 | 2238.0 | — | AVGHLDDLPGAL SALSDLHAHK |
| αT10 + 11 | 91–99 | 22.3 | 1105.3 | 1105.1 | — | IRVDPVNFK |
| αT11 | 93–99 | 20.9 | 817.9 | 817.8 | — | VDPVNFK |
| αT13 | 128–139 | 25.1 | 1279.5 | 1279.9 | 1.757 | FLANVSTVLTSK |
| αT14 | 140–141 | 17.6 | 337.4 | 337.4 | — | YR |
| βT1 | 1–8 | 19.35 | 912.0 | 912.1 | 1.641 | VHLSAEEK |
| βT2 | 9–17 | 20.7 | 972.1 | 972.3 | — | EAVLGLWGK |
| βT3 | 18–30 | 26.1 | 1314.4 | 1314.5 | 1.467 | VNVDEVGGEAL GR |
| βT4 | 31–40 | 24.2 | 1274.5 | 1274.8 | 2.314 | LLVVYPWTQR |
| βT5 | 41–59 | 28.3 | 2046.2 | 2046.7 | — | FFESFGDLSNADA VMGNPK |
| βT6 + 7 | 60–65 | 20.0 | 656.8 | 656.7 | 2.101 | VKAHGK |
| βT8 + 9 | 66–76 | 27.5 | 1239.4 | 1239.0 | 1.670 | KVLQSFSDGLK |
| βT9 | 67–76 | 21.2 | 1093.2 | 1093.5 | 2.485 | VLQSFSDGLK |
| βT10 | 77–82 | 20.1 | 738.8 | 738.6 | — | HLDNLK |
| βT11 | 83–87 | 17.0 | 522.6 | 522.5 | 1.463 | GTFAK |
| βT12 | 88–104 | 29.7 | 2052.2 | 2051.7 | — | LSELHCDQLHVD PENFR |
| βT13 | 105–116 | 25.5 | 1265.6 | 1265.4 | — | LLGNVIVVVLAR |
| βT15 | 118–132 | 25.7 | 1686.8 | 1687.3 | — | LGHDFNPDVQAA FQK |
| βT16 | 133–144 | 22.5 | 1149.4 | 1149.2 | 1.692 | VVAGVANALAK |
| βT17 | 145–146 | 17.6 | 318.3 | 318.4 | — | YH |

[a]Average mass of all charge states of the fragment observed.

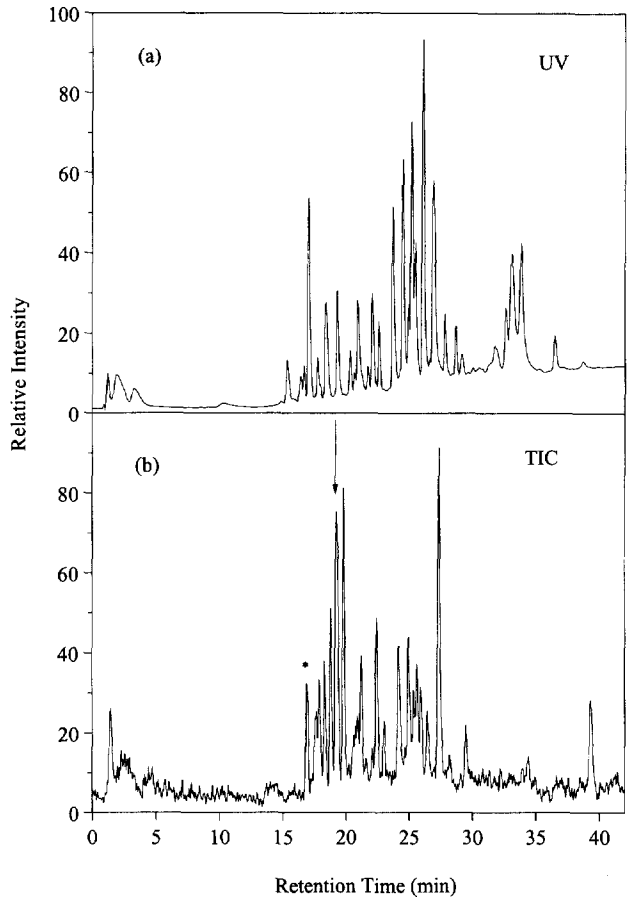


**Figure 2.** Capillary HPLC separation of tryptic digest of pig hemoglobin with 30 pmol sample injection (**a**) UV trace; (**b**) TIC.

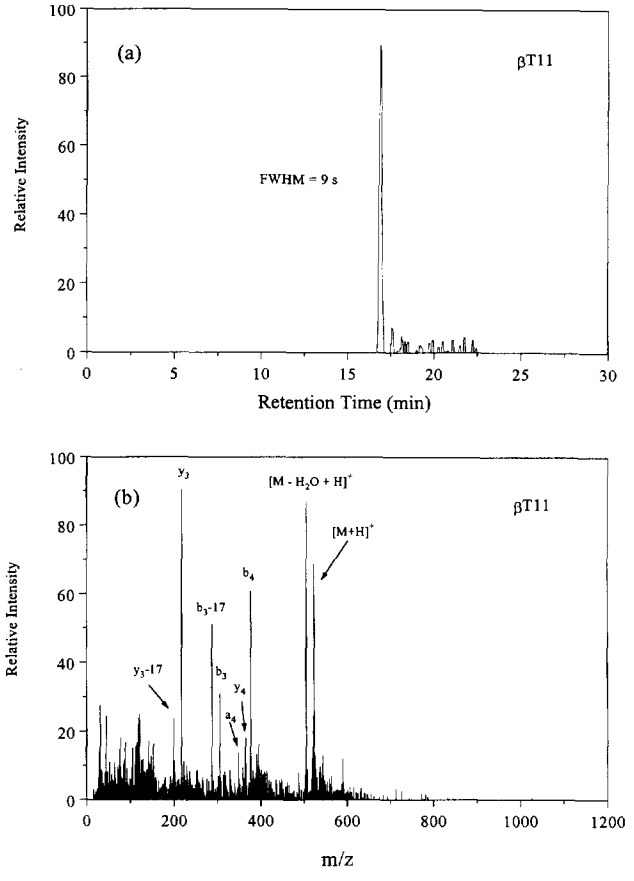


**Figure 3.** (**a**) Extracted ion profile of βT11 fragment; (**b**) MS/MS spectrum of βT11 fragment. Singly charged ion of βT11 was selected as the precursor ion.

be reduced by half, because each cycle now contains the acquisition of only one spectrum rather than two spectra as in the previous version of our optimization routine.

In Figure 1 are shown the total ion chromatogram (TIC) and MS/MS spectra of bradykinin eluted from a capillary HPLC column. Figure 1b was obtained by using the previous data analysis scheme employing comparison of the intensity ratio obtained from two spectra. Figure 1c was obtained by using the new data analysis scheme employing comparison of the intensity ratio obtained from one spectrum. The same amount of sample (~10 pmol) and the same separation conditions were used in these two experiments, where the TIC corresponding to this sample injection is shown in Figure 1a. The MS/MS spectrum shown in Figure 1c has a higher quality in terms of signal-to-noise (S/N) ratio and fragmentation patterns. Only 3.5 s were used to optimize the tickle voltage in Figure 1c, while 8 s were used in Figure 1b. Because the FWHM of the peak shown in Figure 1a is about 8 s, a significantly larger portion of the peak has been used to obtain the MS/MS spectrum in Figure 1c, resulting in a higher S/N ratio. Also, a slightly improved fragmentation pattern was observed in Figure 1c. This is probably due to the fact that the optimization of the tickle voltage in Figure 1c was virtually independent of the elution profile of the peak. As the peak elutes from an HPLC column, its concentration could change considerably, resulting in differences in precursor ion intensity between two contiguous spectra even with the same tickle voltage. This could introduce a bias in terms of obtaining the optimal tickle voltage if the intensity ratio of the molecular ions in two contiguous spectra is used as a measurement of the fragmentation efficiency.

The repeatability of this system to optimize the tickle voltage has been tested. The optimized tickle voltages for bradykinin with 3 consecutive injections containing different sample amounts are listed in Table 2. Tickle voltages with good precision were obtained with these experiments. As the peak width decreased below ~7 s, a repeatable tickle voltage was still achieved even though the S/N of the MS/MS spectrum was relatively poor due to the inadequate time to collect spectra at the optimal tickle voltage.

## *LC/MS/MS Analysis of Hemoglobin Digest*

The capability of the IT/reTOF-MS as an on-line tandem mass detector for capillary HPLC separations was

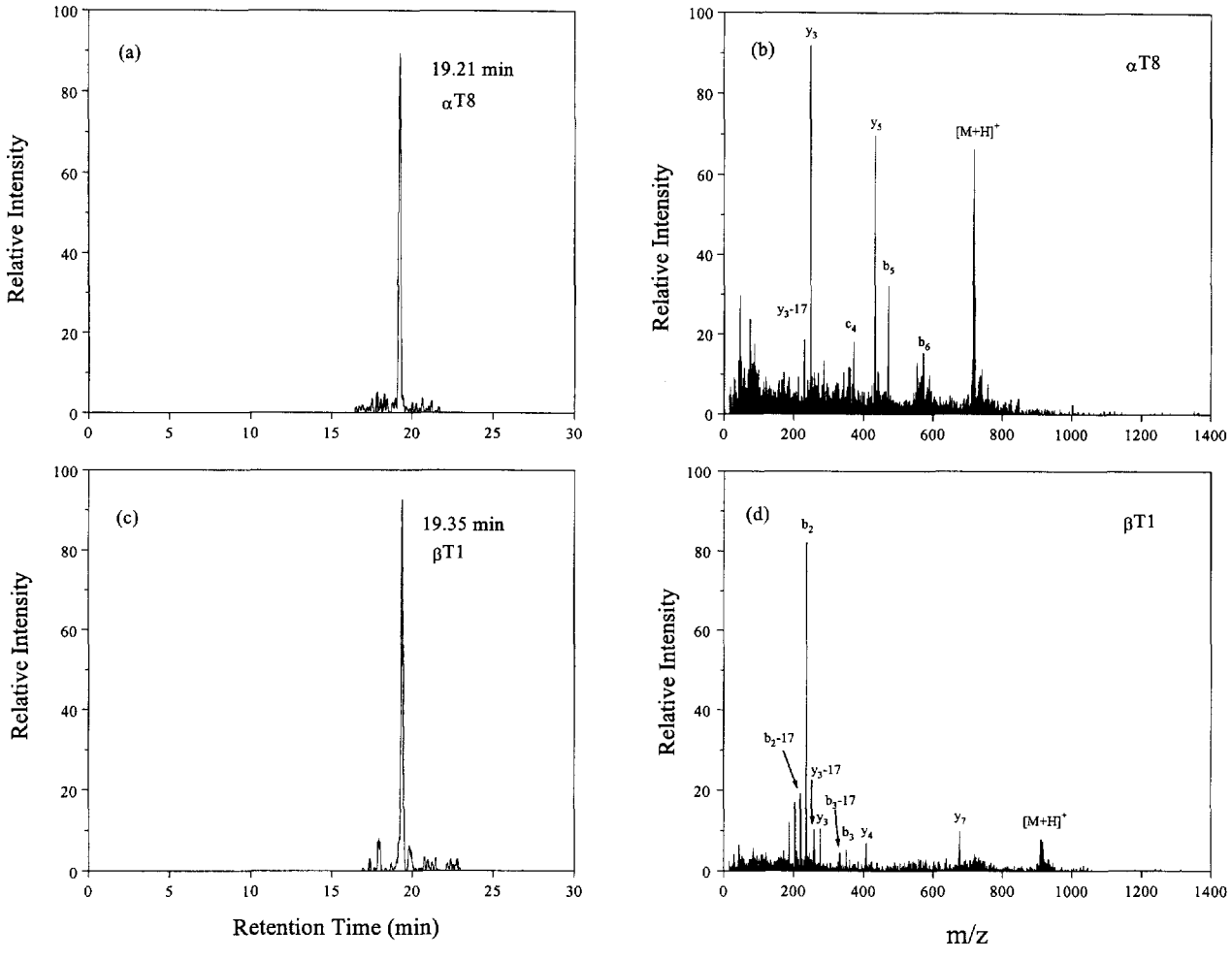


**Figure 4.** (a) Extracted ion profile of αT8 fragment; (b) MS/MS spectrum of αT8 fragment; (c) Extracted ion profile of βT1 fragment; (d) MS/MS spectrum of βT1 fragment. Singly charged ion was selected as the precursor ions.

demonstrated in the analysis of a tryptic digest of pig hemoglobin. Hemoglobin is a relatively large protein that consists of two subunits (α chain and β chain) with a molecular weight of ~30 kDa. A tryptic digestion of hemoglobin results in about 30 fragments. In Figure 2 are shown the UV trace and TIC of the capillary HPLC separation of a hemoglobin digest. As a result of the high separation efficiency of capillary HPLC, most of the peptides resulting from tryptic digestion have been resolved. The TIC was qualitatively similar to the UV trace, indicating the mass detector could accurately record a complex separation profile. Some peaks between retention times of 30–37 min in the UV trace are missing in the TIC. This is probably because the molecular weight of these components are larger than the mass range set in these experiments. In these experiments, a sampling $m/z$ range of ~150 to 1500 was used, which is usually sufficient for protein digest analysis. A wider mass range is possible for the mass spectrometer but is limited by our data system, because that would increase the data file size and slow down the data transfer and storage process. With this preset $m/z$ range, components with $m/z$ over 1500 were not detected. In order to detect relatively large components, they must readily form multiply charged ions that apparently was not the case in this particular experiment.

After this initial LC/MS analysis, each chromatographic peak was correlated to one or more masses. The measured and the calculated masses and their corresponding amino acid sequences are listed in Table 3. The peak widths of most of these peaks were relatively narrow, with FWHM around 10 s. With a rapid spectral acquisition speed and a fast tickle voltage optimization scheme, the IT/reTOF-MS could successfully obtain the MS/MS spectra for multiple components within one LC/MS/MS run. One of these MS/MS spectra is shown in Figure 3b as an example. This MS/MS spectrum was obtained from the peak marked by an asterisk (*) in Figure 2b. The extracted ion profile of this component shown in Figure 3a indicated that the FWHM of this peak was only about 9 s, nevertheless, the MS/MS spectrum shown in Figure 3b exhibited excellent S/N ratio and fragmentation information.

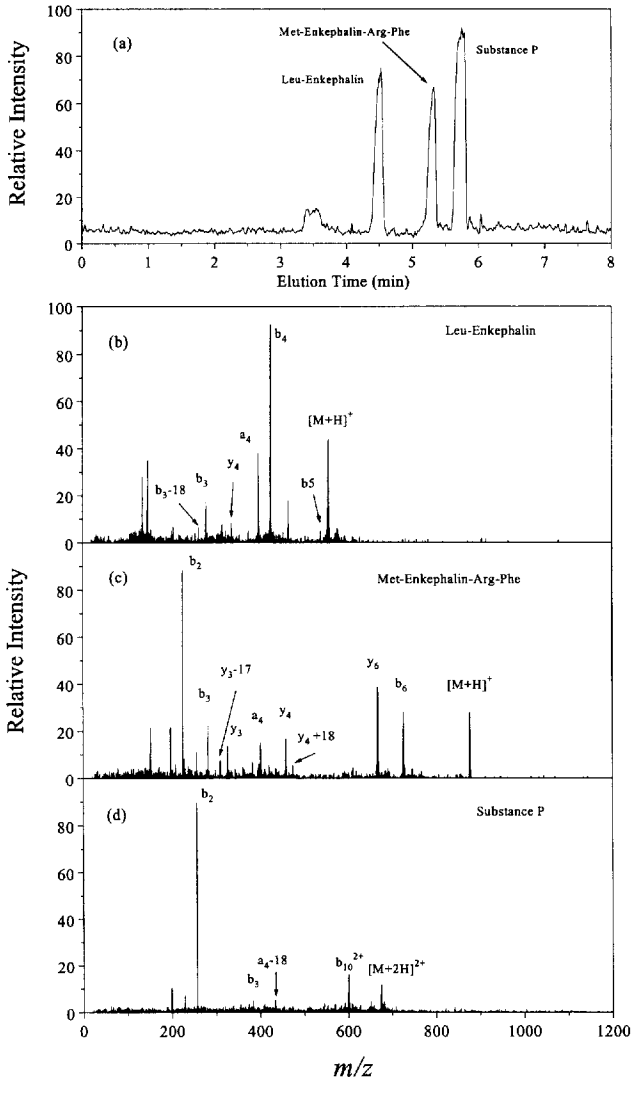


**Figure 5.** (**a**) Total ion electropherogram of CE/MS analysis of a three peptide mixture; (**b**) MS/MS spectrum for leucine-enkephalin. Singly charged ion was selected as the precursor ion; (**b**) MS/MS spectrum for methionine-enkephalin-arginine-phenylalanine. Singly charged ion was selected as the precursor ion; (**c**) MS/MS spectrum for substance P. Doubly charged ion of substance P was selected as the precursor ion.

It should be noted that most of the peaks shown in the TIC (Figure 2b) were eluted with retention times between a relatively narrow period (17 to 30 min), resulting in a number of closely eluting or even co-eluting components. The peak marked by an arrow in Figure 2b actually contained two co-eluting components. Their extracted ion profiles shown in Figures 4a and 4c indicated their retention times only differed by about 8 s with peak widths (FWHM) of around only 10 s each. The elution conditions provide a challenge for a tandem mass detector if the MS/MS spectra for both components are to be obtained within one run. With the rapid sampling rate and the fast optimization scheme on the IT/reTOF-MS, the MS/MS spectra for these two components could be obtained within one LC/MS/MS run. These spectra are shown in Figures 4b and 4d. Figure 4b was obtained by collecting averaged spectra for 8 s after the tickle voltage was optimized, and Figure 4d was obtained by averaging spectra for 7 s.

### *CE/MS/MS Analysis of a Peptide Mixture*

Compared to HPLC, CE has a much higher separation efficiency, and this high efficiency can be achieved in a very short analysis time. As a result, the peaks in CE are extremely narrow, usually on the order of several seconds, making on-line MS/MS detection especially difficult. In Figure 5a is shown the total ion electropherogram (TIE) of a CE separation of a peptide mixture, containing leucine-enkephalin, methionine-enkephalin-arginine-phenylalanine, and substance P. Although the FWHM of these three peaks were all less than 10 s, relatively high quality MS/MS spectra for all the three components were obtained within one CE/MS/MS run. These MS/MS spectra are shown in Figures 5b–d.

In the above experiments, a relatively large amount of sample was injected (~50 fmol) that resulted in some band broadening due to slight overloading of the capillary. The resulting peaks were relatively broad compared with our previous experiments on CE/MS [18]. This was done in order to improve the quality of the MS/MS spectra. Currently, a 2 Hz spectral acquisition speed has been used in all the on-line MS/MS experiments. Because the tickle voltage usually needs to be adjusted around eight times before it reaches the optimal value, 4 s are required for this period after the threshold of a peak is detected. In order to obtain an MS/MS spectrum with reasonable S/N ratio, at least several seconds are required to collect the averaged spectra. Therefore, a minimum FWHM of about 5 s for MS/MS is required on our current system by using a 2 Hz sampling rate. One method to increase the capability of our system to achieve fast separations is to increase the sampling rate in the MS/MS mode. At this point, a practical limit to further increasing this speed is the hardware used to generate and load the isolation and activation waveforms [18]. Therefore, achieving a faster spectral acquisition speed is possible when the AWFG and the computer hardware are upgraded. We have already demonstrated sampling rates of 10 Hz for conditions not involving isolation and activation [22]. The ultimate spectral acquisition speed is limited, however, by the duty cycle required and the total time needed to perform the ion isolation and activation.

## Conclusions

In this work, the capabilities of the IT/reTOF-MS as a tandem mass detector for on-line separations have been explored. The rapid MS/MS spectral acquisition speed, combined with a fast data-dependent data acquisition scheme, allowed for obtaining MS/MS spectra of sharp and closely eluting components from on-line capillary

separations. This was obtained by using a methodology where the intensity ratio of the product ions over the molecular ions within one spectrum was employed as the measurement for tickle voltage optimization. As a result, a hemoglobin digest was analyzed on this system and MS/MS spectra for multiple tryptic fragments have been obtained within one LC/MS/MS run. In addition, a CE separation of a peptide mixture has been analyzed in the on-line MS/MS mode and the MS/MS spectra of all the components could be obtained. Even though the peak widths of the CE/MS separation are not much sharper than those in the LC/MS experiments, it still provided more of a challenge in obtaining optimal tickle voltages with high precision due to the relatively low stability of the nanospray source used in the CE/MS experiments.

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